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Tar DNA-binding protein-43 (TDP-43) regulates axon growth *in vitro* and *in vivo*

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ABSTRACT

Intracellular inclusions of the TAR-DNA binding protein 43 (TDP-43) have been reported in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD-TDP). Rare mutations in *TARDBP* have been linked to both ALS and FTD-TDP suggesting that TDP-43 dysfunction is mechanistic in causing disease. TDP-43 is a predominantly nuclear protein with roles in regulating RNA transcription, splicing, stability and transport. In ALS, TDP-43 aberrantly accumulates in the cytoplasm of motor neurons where it forms aggregates. However it has until recently been unclear whether the toxic effects of TDP-43 involve recruitment to motor axons, and what effects this might have on axonal growth and integrity. Here we use chick embryonic motor neurons, *in vivo* and *in vitro*, to model the acute effects of TDP-43. We show that wild-type and two TDP-43 mutant proteins cause toxicity in chick embryonic motor neurons *in vivo*. Moreover, TDP-43 is increasingly mislocalised to axons over time *in vivo*, axon growth to peripheral targets is truncated, and expression of neurofilament-associated antigen is reduced relative to control motor neurons. In primary spinal motor neurons *in vitro*, a progressive translocation of TDP-43 to the cytoplasm occurs over time, similar to that observed *in vivo*. This coincides with the appearance of cytoplasmic aggregates, a reduction in the axonal length, and cellular toxicity, which was most striking for neurons expressing TDP-43 mutant forms. These observations suggest that the capacity of spinal motor neurons to produce and maintain an axon is compromised by dysregulation of TDP-43 and that the disruption of cytoskeletal integrity may play a role in the pathogenesis of ALS and FTD-TDP.

Introduction

Amyotrophic lateral sclerosis (ALS) is a late-onset, relentlessly progressive neurodegenerative disease. TDP-43 is the major protein present in neuronal and glial inclusions in both sporadic and familial forms of ALS and tau-negative frontotemporal dementia (FTD-TDP), (Forman et al., 2007; Mackenzie et al., 2007; Neumann et al., 2006). We, and others, have shown that mutations in the C terminal domain of the gene encoding TDP-43 (*TARDBP*) are causally linked to familial and sporadic ALS and FTD-TDP (Blair et al., 2010; Chio et al., 2009; Corrado et al., 2010; Damme et al., 2010; Drepper et al., 2011; Gitcho et al., 2008; Kabashi et al., 2008; Kwiatkowski et al., 2009; Rutherford et al., 2008; Sreedharan et al., 2008; Van Deerlin et al., 2008; Vance et al., 2009; Yokoseki et al., 2008). Intriguingly, TDP inclusions are also found in a smaller proportion of patients with Alzheimer's and Parkinson's disease, but their role in the pathogenesis is uncertain (Nakashima-Yasuda et al., 2007).

TDP-43 is a predominantly nuclear protein with DNA and RNA binding properties. It has multiple roles in RNA-processing that are crucial in early development, as genetic deletion of TDP-43 is embryonic lethal between embryonic days 3 and 8 (Chiang et al., 2010; Kraemer et al., 2010; Sephton et al., 2010; Wu et al., 2010). TDP-43 can act as a transcriptional repressor and is implicated in exon skipping/retention, RNA transport and stability and micro RNA biogenesis (Casafont et al., 2009; Strong et al., 2007). In ALS and FTD-TDP, prominent TDP-43 aggregates are present in the cytoplasm of surviving neurons, which often show clearing of the protein from the nucleus (Neumann et al., 2006; Vance et al., 2009). However, it remains unclear whether the gain or loss of TDP-43 function is mechanistic in disease. In cellular and some animal models overexpressing TDP-43, deletion of the RNA binding and C terminal domains was required for toxicity rather than cytoplasmic mislocalisation (Voigt et al., 2010; Wegorzewska et al., 2009). It is possible that both the loss of nuclear TDP-43 and its sequestration in the cytoplasm lead to defective RNA processing (Lagier-Tourenne et al., 2009; Strong MJ, 2010). The extent to which TDP-43 is localised in axons has been uncertain, but a recent study shows its recruitment to motor axons, where it co-localises with a number of

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mRNA-binding proteins (Fallini et al., 2012). Given that TDP-43 binds to nearly one third of all neuronal RNA transcripts, its effects could impact a very wide array of cellular pathways, many of which are critical in neuronal development including components of the cytoskeleton (Polymenidou et al., 2011; Tollervey et al., 2011a, 2011b). For example, TDP-43 interacts directly with the 3′ UTR of the low molecular weight (68 kDa) neurofilament mRNA (NFL) (Strong et al., 2007), stabilising it and preventing its degradation. Conversely, in ALS the expression of the NFL mRNA is suppressed and it is preferentially sequestered to stress granules (Strong et al., 2007; Volkening et al., 2009).

We have previously shown that *in vivo* over-expression of TDP-43 mutant forms in the chick spinal cord analysed over short time intervals (24 h) leads to cell death and developmental delay (Sreedharan et al., 2008). Here we investigate the effects of TDP-43 mutant and wild-type forms on motor neuron integrity and axon outgrowth *in vitro* and *in vivo* over a longer period of time, using the chick embryo model. *In vivo*, we found that spinal motor neurons over-expressing TDP-43 (wild-type and mutant) showed a progressive increase in cytoplasmic and axonal TDP-43 mislocalisation over time. Those neurons expressing mutant TDP-43 were more likely to display prominent nuclear aggregates. A down-regulation of neurofilament-associated antigen (NAA) was observed in axons expressing mutant TDP-43, accompanied by de-fasciculation and truncation of the axon projections. When the same constructs were expressed in dissociated E6 spinal motor neurons, TDP-43 aggregation was observed along with a reduction in axon outgrowth. This was more prominent in cells expressing mutant forms than in those expressing wild-type TDP-43. Taken together, our data suggest that over-expression of TDP-43 (wild-type and mutant) leads to cytoplasmic and axonal mislocalisation of TDP-43 in motor neurons, and an impairment of axon growth.

Materials and methods

Plasmids

The N-terminal HA and C-terminal Myc constructs were cloned as described previously (Sreedharan et al., 2008; all cloned into the *pCI-neo* vector). For EGFP-tagged TDP-43 WT and mutant plasmids, the cDNA insert was cloned into the pEFGP-C1 (N-terminal GFP) vector at the XhoI and BamHI sites. Mutant forms of TDP-43 were those containing either the M337V familial mutation, or the Q331K sporadic mutation. Direct sequencing was used to confirm clone alignment and mutagenesis.

Embryos

Pathogen-free white Leghorn hens' eggs were incubated at 39 °C and 70% humidity. Embryos were staged according to Hamburger and Hamilton (1951) and electroporated at HH stage 14 (E2.5).

In ovo electroporation

In ovo electroporation was used to over-express the genes of interest in one half of the spinal cord. On the day of electroporation a small window was cut into the shell to expose the embryo. PBS containing 1% Penicillin–Streptomycin (Invitrogen) was added to keep the embryos moist. Plasmid DNA constructs were dissolved in ddH_2O to give a final concentration of 4.0 μg/μl. In all the experiments, *pEGFPC1* expression vector was used to over-express EGFP as a control. Visualisation of DNA during injection was aided by adding 0.5 μl of 10 mg/ml fast green to 10 μl of DNA solution. A modified method of Momose et al. (1999) was used, as previously described (Sreedharan et al., 2008). Eggs were reincubated for 24–96 h, after which surviving embryos were dissected and fixed using 4% paraformaldehyde for 1–2 h at room temperature.

Cryosections

Fixed embryos were washed with PBST (PBS (Invitrogen) + 0.1% Triton X-100 (Sigma)) 3 times for 30 min each at room temperature and processed through ascending concentrations of sucrose (Sigma) (10%, 20%, 30%) for 30 min each at room temperature. They were finally washed in 1:1 30% Sucrose:OCT and then in OCT (CellPath Ltd.) for 30 min each at room temperature. Embryos were then embedded in OCT, flash-frozen in iso-Pentane (Sigma) using liquid nitrogen and sectioned using a cryostat (Leica Microsystems) maintaining a thickness of 20 μm.

Immunofluorescence

Frozen sections or cells (adhered to glass cover slips) were washed in PBS, permeabilised using 0.5% and 0.05% PBST, respectively (1× PBS + 0.5% Triton X-100). Sections or cells were then blocked using 10% FCS/PBS for 1 h and incubated with primary antibodies overnight at 4 °C (rabbit anti-HA, 1:1000, Sigma; mouse anti-myc, 1:1000, NEB; mouse anti-islet1/2 (4D5), 1:200, DSHB; mouse anti-neurofilament associated protein (3A10), 1:200, DSHB; chicken anti-GFP, 1:1000, Abcam). Sections or cells were washed again with PBS, 3 times for 5 min each, before incubation with fluorescent secondary antibodies for 1–2 h at room temperature (anti-rabbit Alexa Fluor 488; anti-mouse Alexa Fluor 568; anti-mouse Alexa Fluor 488, anti-chicken Alexa Fluor 488, all 1:1000, all Invitrogen). After further washing, sections or cells were mounted using Hardset Vectashield with DAPI (Vector Laboratories). Images were captured using an Axiovert 4.0 (Carl Zeiss) microscope with Image-Pro Express 6.0 (Media Cybernetics). Adobe Photoshop 7.0 (Adobe Systems, Inc.) was used to process the images.

Tunel

Apoptotic cell death was visualized on frozen sections according to the manufacturer's protocol by nuclear DNA fragmentation analysis using a DeadEnd Colorimetric TUNEL System (Promega Corporation, USA). The number of apoptotic cells on the transfected and untransfected sides of the embryo spinal cords was counted and the data were inserted into GraphPad Prism to obtain the graphs. A total of 50–150 embryos were electroporated for each construct and the data were analysed from 10 to 20 sections across at least 6 embryos from 3 sets of experiments.

Neurite length quantification

ImageJ software was used for quantification of neurite length. Cells labelled with anti-GFP and anti-neurofilament antibodies were imaged using an Axiovert camera (Volocity software was used for image processing) at 10× magnification. Images were then used to analyse the total length of the neurites, length of longest and shortest neurites and number of neurite branches using the NeuronJ plugin of ImageJ. A total of ~150 cells per condition were analysed from 3 independent experiments.

Spinal motor neuron cultures

Glass coverslips were coated with poly-D-ornithine and laminin (15 μg/ml and 1 mg/ml, respectively; Sigma). Motor neuron cultures were performed as previously described (Murray et al., 2010). For preparation of spinal motor neurons, we used the ventral portion of the cervical and thoracic spinal cord, which is enriched in motor neurons at HH stages 28–29 (E6). Dissociated neurons were plated on laminin-coated coverslips, in Neurobasal medium containing 2% (v/v) B27 supplement, 2% (v/v) horse serum, 0.1% (v/v) β-mercaptoethanol, 0.35% (v/v) Glutamax, 1% (v/v) penicillin/streptomycin (all from Gibco), 1% (v/v)

chick embryo extract and 50 ng/ml CNTF (R&D Systems). Neurons were transfected with *EGFP-WT*, *EGFP-Q*, *EGFP-M* or *pEGFP-C1* construct as a control, using the chick nucleofection kit and an Amaxa machine (Lonza). The efficient transfection of these constructs was determined by immunostaining using anti-GFP antibodies.

Rat cortical neuron cultures

Rat cortices were obtained at embryonic day 18. Upon dissection, the cortical tissues were dissociated by digestion with Trypsin (Worthington Biochemical) in Hanks buffer for 15 min and then transferred to Neurobasal medium (Gibco) with 2% B-27, 2% FBS, 1% glutamax and 1% penicillin–streptomysin. The dissociated neurons were then transfected with different plasmids (Control GFP and TDP-43 wild-type and mutants) at a concentration of 2 μg or 4 μg of DNA per 1 million cells using the Amaxa transfection kit and then plated at a density of 140,000 cells in 35 mm confocal dishes (Thistle scientific) for western blotting. The confocal dishes were previously coated with poly-D-lysine and laminin (1 mg/ml, Sigma). The cells were maintained in Neurobasal medium supplemented with B-27, penicillin–streptomysin and glutamax and incubated at 37 °C with 5% CO_2. Cells were then lysed and stored at −20 °C for western blotting.

Western blots

Proteins were separated by SDS-PAGE and transferred onto a Hybond-P PVDF membrane (GE Healthcare) by wet transfer, incubated with rabbit anti-TDP-43 antibody (10782-2-AP, Proteintech) or mouse anti-GAPDH antibody (Sigma) followed by HRP-conjugated anti-rabbit or anti-mouse secondary antibody (Dako). 43 kD and 70 kD bands were detected using anti-TDP-43 antibody representing the endogenous TDP-43 and exogenous EGFP-tagged TDP-43 respectively. In parallel, the same samples were blotted using anti-GAPDH antibody. Blots were developed using luminescent substrates (Roche). Bands were quantified densitometrically using ImageJ software (National Institute of Health) and standardised for each lane with respect to GAPDH.

Results

TDP-43 causes neurotoxicity in the chick embryo spinal cord

In order to gain insights into the effects of TDP-43 wild-type and mutant forms on motor axons *in vivo*, we electroporated constructs tagged with N-terminal HA and C-terminal Myc into the chick spinal cord at embryonic day 2.5 (E2.5). Mutant forms of TDP-43 were those containing either the M337V familial mutation, or the Q331K sporadic mutation, which will be referred to as the M or Q mutant respectively. In previous electroporation experiments in the chick spinal cord, we have shown that transfection of 5–10% of cells in the chick embryo spinal cord with Q and M mutant constructs tagged with Myc/HA resulted in a significant increase in the number of apoptotic cells as compared to wild-type TDP-43 transfection (Sreedharan et al., 2008). We have further optimised the transfection conditions such that with higher concentrations of DNA we obtain higher levels of transfection, up to 50–80% cells in the chick embryo spinal cord; an EGFP plasmid was electroporated as a control (Fig. 1A). Embryos were reincubated for 24 h until E3.5, cryo-sectioned and immunostained using anti-GFP antibodies for the EGFP controls, or with anti-Myc and anti-HA antibodies in

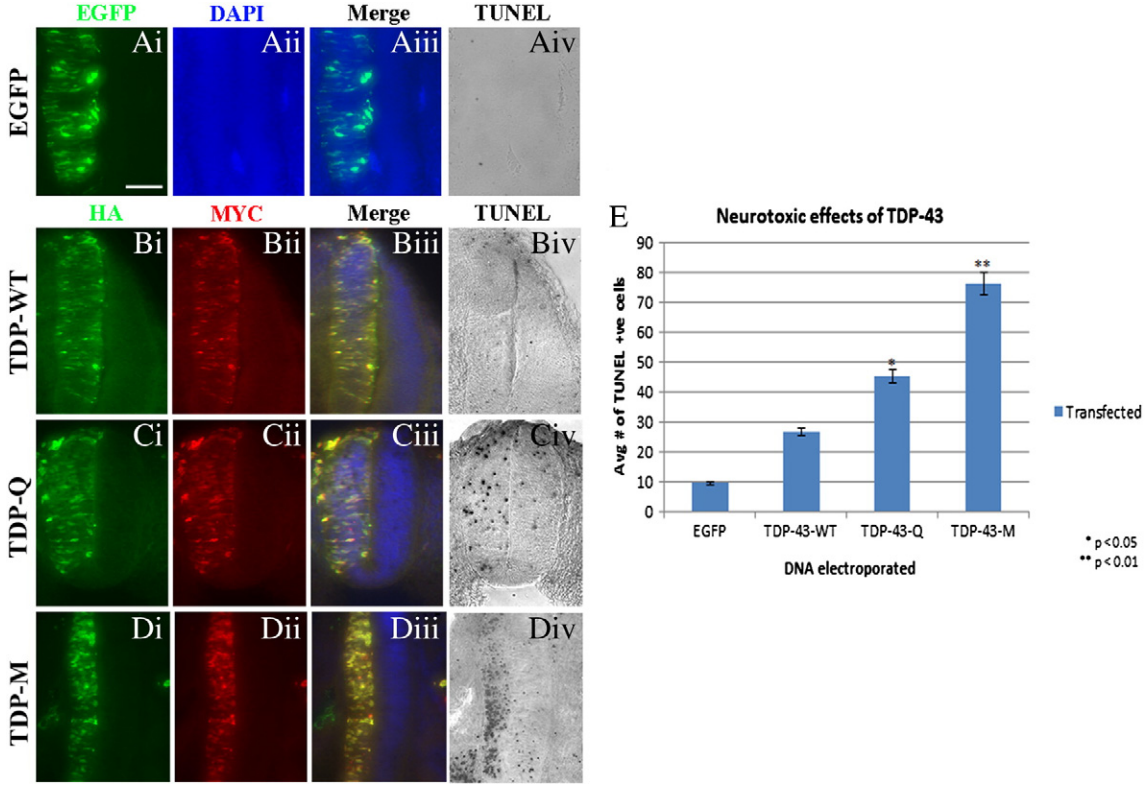

Fig. 1. Effects of over-expression of *EGFP*, *HA/Myc-WT*, *HA/Myc-Q* and *HA/Myc-M* TDP-43 isoforms in the chick embryo spinal cord. Representative transverse sections from embryos electroporated with *EGFP* (A) or *TDP-43* isoforms *HA/Myc-WT* (B), *HA/Myc-Q* (C) and *HA/Myc-M* (D). Panel Ai (green) labelled with anti-GFP; Panel Aii (blue) labelled with DAPI; Panel Aiii (overlay yellowish) merge of Panels Ai and Aii; Panels Bi–Di, (green) labelled with anti-rabbit anti-HA; Panels Bii–Dii (red) labelled with anti-mouse anti-myc and Panels Biii–Diii (DAPI (blue) with HA and MYC overlay (yellow/red)) show the merged images. Panels Aiv–Div show sections stained for apoptotic nuclei (TUNE-stained nuclei appear as black dots). The spinal cords electroporated with EGFP (Aiv) or *HA/Myc-WT* (Biv) show very few TUNEL positive cells compared to embryos electroporated with *HA/Myc-Q* (Civ) and *HA/Myc-M* (Div) which show frequent TUNEL-positive nuclei on the transfected side of the spinal cord. (E) Graph representing the average number of TUNEL + ve cells after electroporation of EGFP, *HA/Myc-WT*, *HA/Myc-Q* and *HA/Myc-M*. A total of 122 embryos were electroporated for each construct and the data was analysed from 60 sections across at least 11 embryos from 3 sets of experiments. The expression of Myc/HA was widespread in the spinal cord. Scale bar = 100 μm.

order to determine the pattern of expression of WT and mutant TDP-43 proteins (Figs. 1Ai-iii, Bi-iii, Ci-iii). To determine the number of cells showing apoptosis, TUNEL DNA fragmentation analysis was used. Electroporation of the EGFP control construct alone resulted in a small number of TUNEL + ve cells (Figs. 1Ai–iv). Electroporation of a TDP43 wild-type construct (*WT-TDP43*) resulted in a slight increase in the number of TUNEL + ve cells (Figs. 1; Bi–iv; E) but this was not significantly different from either the EGFP control ($p > 0.05$), or non-electroporated controls (data not shown). Compared with the effects of both these constructs, there was a significant increase in the numbers of apoptotic nuclei in spinal cords electroporated with TDP-43 Q or M mutant constructs (Figs. 1Ci–Div, E; $p < 0.05$ and 0.01 for Q and M vs EGFP respectively). A qualititative comparison of these data showed increased levels of toxicity of WT, Q and M forms relative to our previous study (Sreedharan et al., 2008), in which we had achieved a lower transfection efficiency. Abnormal cleavage of TDP-43 produces phosphorylated C-terminal fragments (CTF's) under pathological conditions. These CTF's are enhanced within neuronal inclusions. Our strategy for using N and C-terminal tagged TDP-43 constructs was to analyse any difference in the localisation of the cleaved TDP-43 protein, however, we did not see any apparent difference as the pattern of HA and Myc staining was closely similar (Fig. 1).

We next electroporated EGFP-tagged TDP-43 WT and mutant forms and compared their toxicity with EGFP controls. These EGFP-tagged TDP-43 constructs produced an EGFP fusion protein in which the GFP fluorescence reproduces the TDP-43 expression pattern (Fig. 2). We observed an increased number of neurons electroporated in the spinal cord *in vivo* compared with previous studies (Fig. 2). Analysis by TUNEL staining demonstrated a significant increase in the number of apoptotic nuclei in embryos electroporated with *Q* and *M* mutant

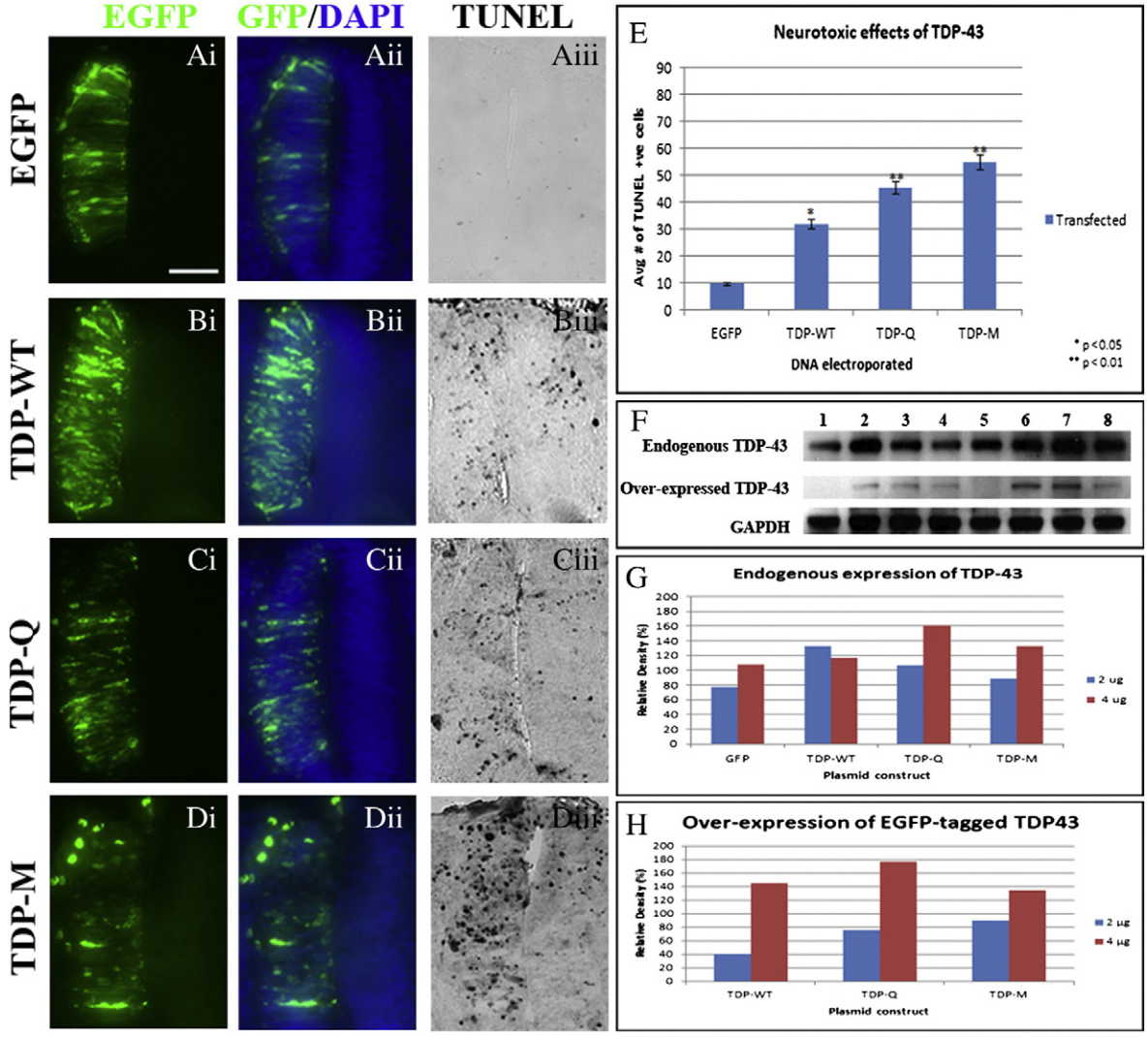

Fig. 2. Effects of over-expression of EGFP, *EGFP-TDP-43^{WT}*, *EGFP-TDP-43^{Q331K}* and *EGFP-TDP-43^{M337V}* TDP-43 isoforms in chick embryo spinal cord. Representative transverse sections from embryos electroporated with *EGFP* (A), or TDP-43 isoforms *WT* (B), *Q* (C) and *M* (D). Panels Ai–Di, EGFP fluorescence (green); Panels Aii–Dii (DAPI (blue) with GFP overlay (green)) show the unilateral expression of TDP-43 EGFP-tagged constructs in the spinal cord. Panel Aiii–Diii show the same sections stained for apoptotic nuclei (TUNEL-stained nuclei appear black). The spinal cords electroporated with *WT* (Biii) show few TUNEL-positive cells compared to embryos electroporated with *Q* (Ciii) and *M* (Diii) which show frequent TUNEL-positive nuclei on the transfected side of the spinal cord. (E) Graph representing the average number of TUNEL + ve cells after electroporation of EGFP, and TDP-43 isoforms *WT*, *Q* and *M*. (F) Western blotsof cortical neuron lysates using anti-TDP-43 antibodies representing endogenous levels of TDP-43 and over-expression of EGFP-tagged TDP-43 WT, Q and M as compared to GAPDH. Lanes 1, 2, 3, 4 represent EGFP, TDP-WT, TDP-Q and TDP-M at 2 µg, respectively, and lanes 5, 6, 7, 8 represent EGFP, TDP-WT, TDP-Q and TDP-M at 4 µg, respectively. (G) Semi-quantitative analysis of endogenous TDP-43 protein expression levels in neuronal lysates. Western blots were analysed using densitometry of the resulting 43 kD band. The levels of endogenous TDP-43 protein expression increase for TDP-43-Q and TDP-43-M relative to EGFP-transfected cells for 4 µg of transfected DNA. (H) Semi-quantitative analysis of EGFP-tagged TDP-43 protein expression levels in neuronal lysates. Western blots using anti-TDP-43 antibodies were analysed using densitometry of the resulting 70 kD band. The levels of protein expression increase with increasing concentrations of transfected DNA plasmid. A total of 25–50 embryos were electroporated for each construct and the data was analysed from 60 sections across at least 11 embryos from 3 sets of experiments. Scale bar = 100 µm.

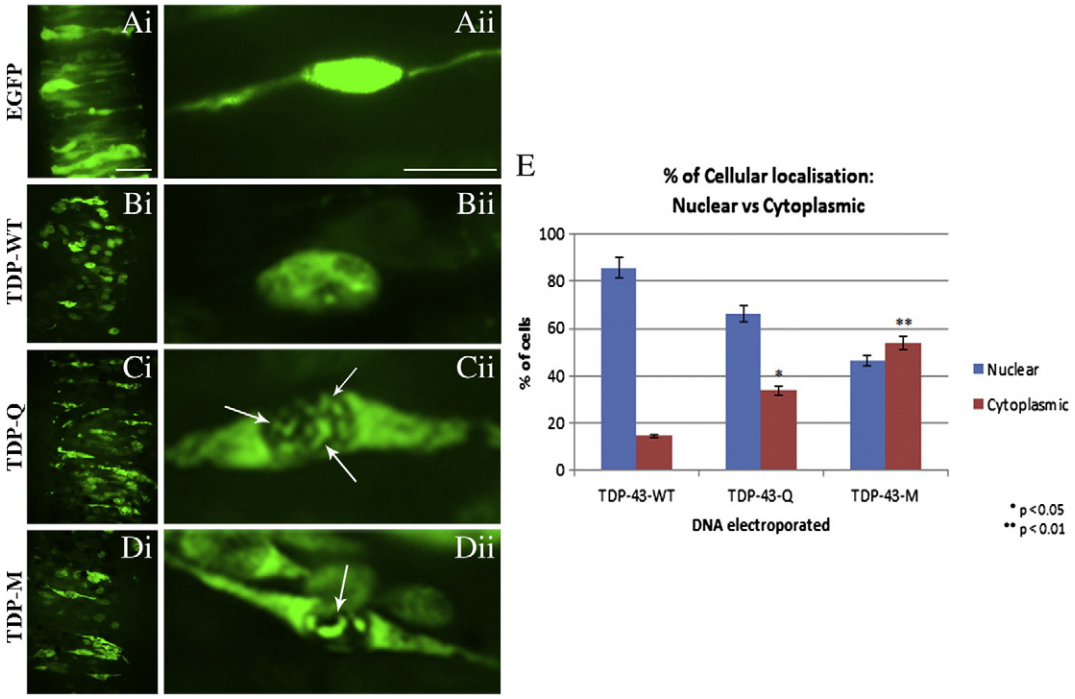

Fig. 3. Cytoplasmic mis-localisation of mutant TDP-43. Representative images from embryos electroporated with *EGFP* (Ai–Aii), or TDP-43 isoforms *WT* (Bi–Bii), *Q* (Ci–Cii) and *M* (Di–Dii). Panels Ai–Di, EGFP fluorescence (green) show the expression of EGFP and TDP-43 EGFP-tagged constructs in the spinal cord; Panels Aii–Dii confocal images of individual cells. Over-expression of mutant TDP-43 causes diffuse cytoplasmic localisation along with nuclear inclusions (arrows). (E) Graph representing the average percentage of cells showing a nuclear vs cytoplasmic localisation after electroporation of *WT*, *Q* and *M*. Asterisks denote significance levels. Scale bar = 100 μm.

constructs compared with those expressing *EGFP* (Figs. 2Ai–iii, Ci–Diii; $p < 0.01$ for comparison with both M and Q mutants; Fig. 2E). There was also a modest but significant increase in the number of apoptotic cells in embryos expressing WT-TDP-43 compared with EGFP controls (Figs. 2Bi–Biii; $p < 0.05$; Fig. 2E). Comparison of the levels of EGFP-tagged TDP-43 from transfections using low or high plasmid

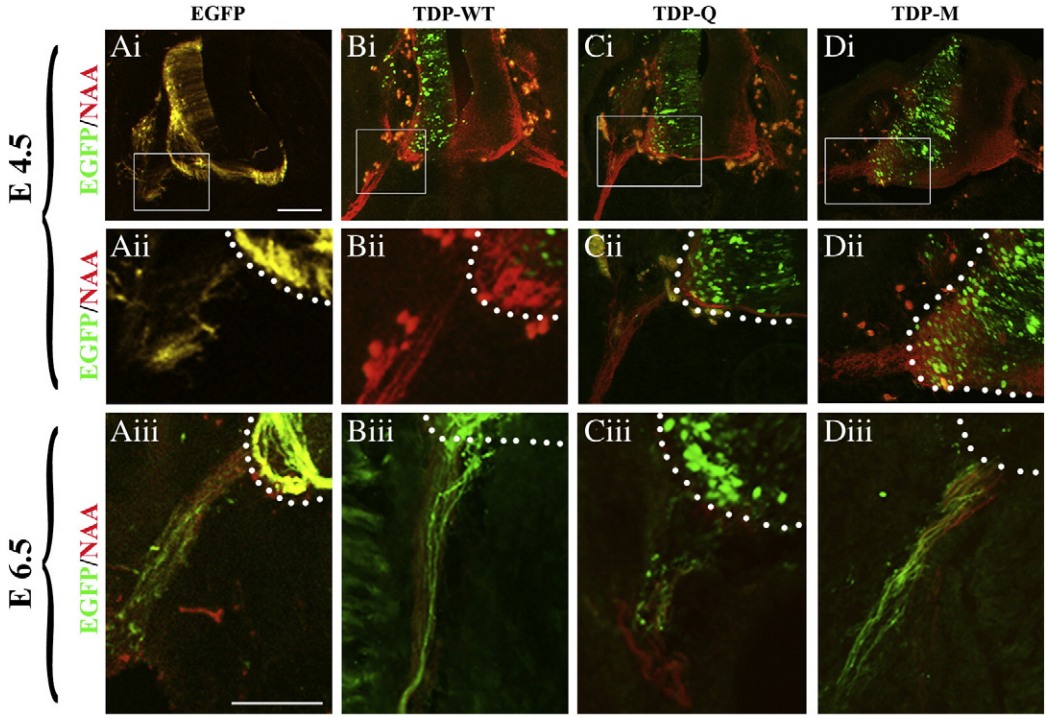

Fig. 4. Axonal mis-localisation of TDP-43 at E6.5 of embryonic development. Representative transverse sections from embryos electroporated with *EGFP* (Ai–Aiii), or TDP-43 isoforms *WT* (Bi–Biii; n = 14), *Q* (Ci–Ciii; n = 12) and *M* (Di–Diii; n = 16). Panels Ai–Di show entire spinal cords demonstrating unilateral expression of EGFP-tagged constructs (green) at E4.5; Panels Aii–Dii represent higher magnification of electroporated sides of the spinal cords of embryos in (Ai–Di), sections are stained for EGFP (green) and neurofilament-associated antigen (NAA; red). Panels Aiii–Diii show higher magnifications of transfected sides of embryos at E6.5. The white dotted line shows the ventral boundary of the neural tube. At E6.5, *WT* (Biii), *Q* (Ciii) and *M* (Diii) isoforms show mis-localisation to the axon projections, as opposed to their localisation in the cell bodies but not in axons as seen in (Bi–Di; Bii-Dii) at E4.5. Scale bar = 100 μm.

concentrations by western blot showed an increase in levels of over-expressed protein with higher concentration of plasmid DNA (Figs. 2F, H). The higher toxicity shown in the current study is therefore most likely due to an increase in the number of neuroepithelial cells transfected using higher plasmid concentrations. In order to assess the effects of over-expressed TDP-43 on endogenous TDP-43, we transfected neurons *in vitro* with EGFP control plasmid and EGFP-tagged TDP-43 WT and mutant plasmids, and performed Western blots using anti-TDP43 antibodies. We observed that over-expression of TDP-43 WT or mutants caused a slight increase in the level of endogenous TDP-43 protein relative to the EGFP control (Fig. 2G). Taken together, these data demonstrate that over-expression of wild-type and mutant TDP-43 causes neurotoxicity in the chick embryo spinal cord, effects which are primarily due to the introduced construct.

Mutant TDP-43 forms nuclear inclusions and mis-localises to the cytoplasm

One of the current hypotheses for the toxicity of TDP-43 is that the mislocalisation of protein in the cytoplasm, leads to cellular dysfunction and degeneration. We therefore tested whether the wild-type or mutant forms of TDP-43 showed cellular mislocalisation *in vivo*. Confocal microscopical analysis at the cellular level was performed on cryosections of embryos, in which the spinal cord had been electroporated at E2.5 and reincubated for 24 h until E3.5 and immunostained as described above. *EGFP*-electroporated control embryos showed 100% cytoplasmic localisation of EGFP signal as expected (Figs. 3, Ai–Aii). Embryos transfected with EGFP-tagged *WT-TDP-43* showed cytoplasmic localisation in 12% of cells (Figs. 3Bi, Bii, E). By contrast, embryos electroporated with EGFP-tagged *Q* and *M TDP-43* showed cytoplasmic mis-localisation in approximately 33% and 52% of cells respectively (Figs. 3Ci, Cii, Di, Dii, E). We also observed the presence of intranuclear inclusions in the embryos electroporated with TDP-43 mutant forms (Figs. 3Cii, Dii). The cytoplasmic mis-localisation of the Q and M mutant proteins was significantly higher than in the wild-type ($p < 0.05$ and $p < 0.01$, respectively). This increase in the cytoplasmic mis-localisation upon electroporation of EGFP-tagged TDP-43 constructs correlates well with the cellular toxicity shown for the mutant forms (Figs. 1 and 2).

TDP-43 shows enhanced cytoplasmic mis-localisation over time in vivo

In order to determine how the subcellular localisation of TDP-43 changed over developmental time, we electroporated EGFP-tagged constructs as above at E2.5 and analysed the embryos at E4.5, E5.5 and E6.5. Cryosections were immunostained using antibodies against EGFP to visualise the distribution of TDP-43 protein, and with antibodies against a neurofilament-associated antigen (NAA) in order to visualise the entirety of spinal motor axon projections. Motor axon projections extending via the ventral roots were NAA-positive at E4.5 and E6.5, showing that the general architecture of axon tracts was preserved (Fig. 4). At all stages analysed, EGFP control protein was localised to the cell bodies and axons which extended out of the spinal cord and into the periphery via the ventral roots (Figs. 4Ai–Aiii; data not shown). This reflects the fact that cytoplasmic EGFP is distributed throughout motor axons following electroporation (e.g. Miyake et al., 2008). By contrast, we observed striking changes in the localisation of EGFP-tagged TDP-43 protein over time by immunostaining with anti-GFP antibodies to detect the TDP-43 protein localisation. At E4.5 and E5.5, the expression of EGFP-tagged WT and mutant TDP-43 was localised to the neuronal cell bodies (as observed by immunostaining using anti-EGFP antibody), with EGFP signal visible up to the edge of the spinal cord (Figs. 4Bi, Bii, Ci, Cii, Di, Dii; data not shown). By contrast, at E6.5, WT, Q and M TDP-43 protein could be detected in motor axons extending via the ventral roots some hundreds of microns away from the spinal cord (Figs. 4Biii, Ciii, Diii; immunostaining for the EGFP tag). Some evidence of apoptosis among neurons expressing WT and mutant forms of *TDP-43* was observed at these stages (data not shown), but the integrity of the motor column overall was preserved (Fig. 4). This suggests that at least a proportion of motor neurons which express TDP-43 wild-type and mutant forms can extend axons along normal pathways *in vivo*, and that a progressive translocation of TDP-43 into motor axons occurs over time.

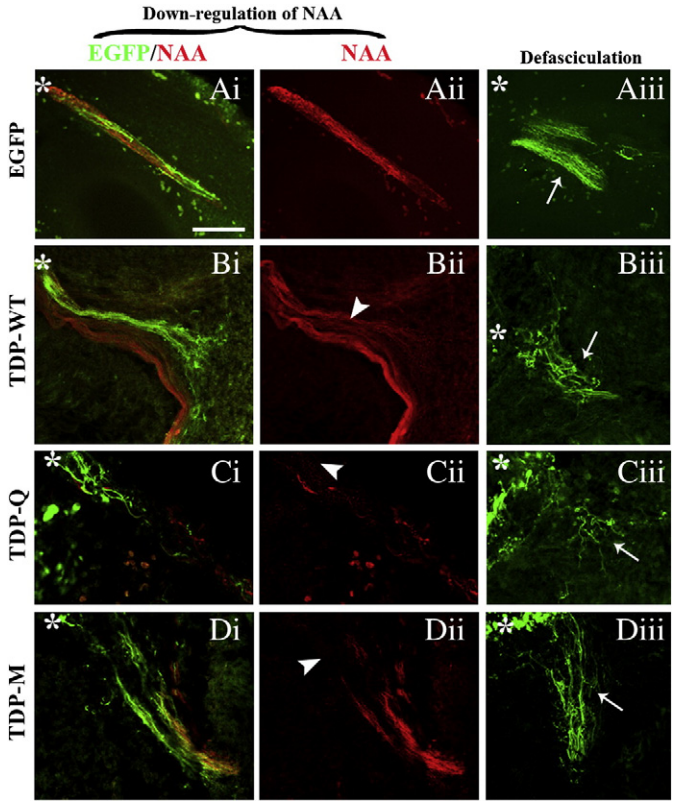

Fig. 5. Axon projection from ventral motor neurons transfected with TDP-43 shows down-regulation of NAA and premature de-fasciculation. Representative confocal images of sections of embryos electroporated with *EGFP* (Ai–Aiii), or TDP-43 isoforms *WT* (Bi–Biii; n = 6), *Q* (Ci–Ciii; n = 6) and *M* (Di–Diii; n = 6). Panels Ai–Di show immunostaining of EGFP (green) and NAA (red), whereas panels Aii–Dii show immunostaining of NAA only. It is evident that upon over-expression of TDP-WT and mutant forms, there is a decrease in NAA immunostaining (arrowheads in Bii–Dii). Panels Aiii–Diii represent immunostaining for EGFP, demonstrating premature defasciculation of axon projections (arrows in Biii–Diii). Asterisks represent the edge of the neural tube. Scale bar = 100 μm.

TDP-43 leads to down-regulation of neurofilament-associated antigen and causes premature truncation and de-fasciculation of axons in vivo

In order to test the molecular effects of wild-type and mutant TDP-43 on the integrity of axon processes, we analysed the expression of NAA in spinal motor axons expressing TDP-43 constructs as analysed at E6.5 (96 hour post electroporation). Immunohistochemistry and confocal imaging showed that there was robust expression of NAA which was widely distributed along the axons of neurons transfected with the EGFP control construct (Fig. 5Ai Aii). However, neurofilament-associated immunoreactivity was greatly reduced in axons which expressed wild-type or mutant TDP-43 (Figs. 5Bi, Bii, Ci, Cii, Di, Dii), suggesting that cytoskeletal components were perturbed by mutant TDP expression. In EGFP-expressing control embryos, motor axons which extended via the ventral roots remained closely bundled together in a fascicle — termed 'fasciculation'. We observed that over-expression of WT and mutant *TDP-43* resulted in debundling or 'defasciculation' of the transfected axons as they extended into the periphery (Figs. 5Aiii–Diii). As these data suggest an effect on the cytoskeletal integrity and pathfinding of axon projections, we also analysed the entirety of these axon projections through serial sections at brachial level, where control EGFP-expressing motor axons were found to project towards the wing

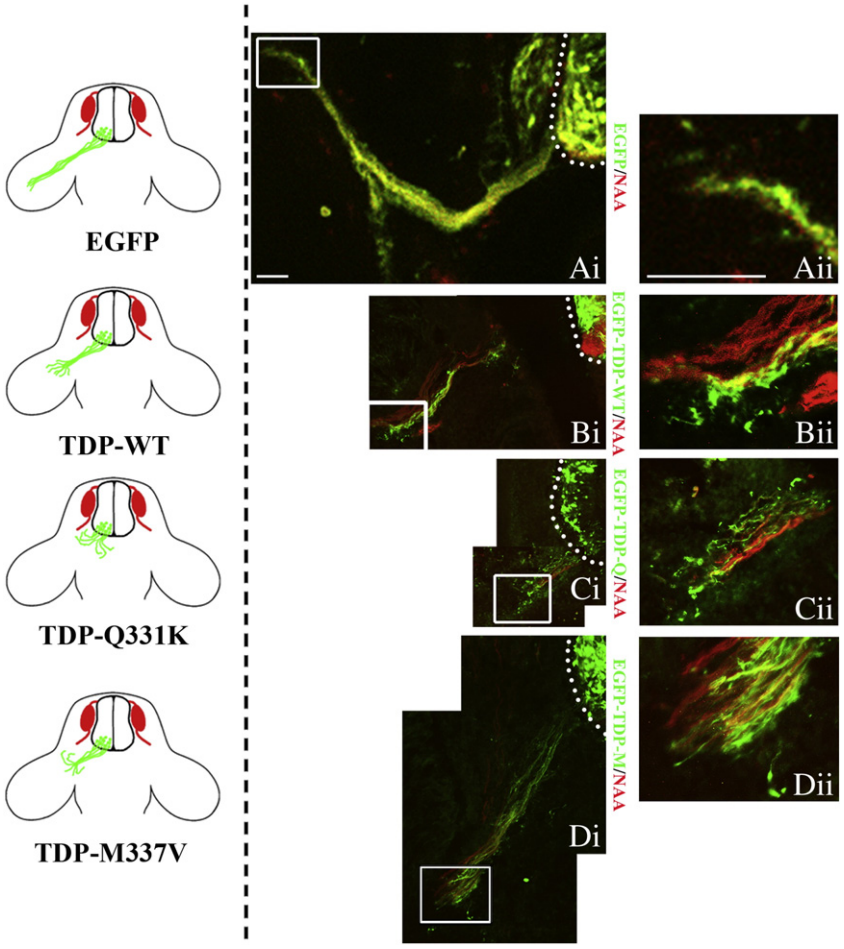

Fig. 6. Axon projections from spinal motor neurons transfected with TDP-43 show aberrant truncation. To left of dotted line are diagrammatic summaries of transverse sections of spinal cord showing phenotypes observed as labelled. To right of dotted line are representative images of sections of embryos electroporated with *EGFP* (A), or TDP-43 isoforms *WT* (B; n = 6), *Q* (C; n = 6) and *M* (D; n = 6). It is apparent that there is aberrant truncation of the axon projections upon over-expression of wild-type or mutant TDP-43 (Ai, Bi, C, Di; boxed region shows truncated axons). (Aii, Bii, Cii, Dii) Magnified images of the boxed regions showing de-fasciculation. Scale bar = 100 μm.

buds (Fig. 6A). A comparison of EGFP-transfected control embryos with wild-type or mutant TDP-43 embryos showed that motor axons were aberrantly truncated, with the truncation most striking for the TDP-43 Q mutant (Figs. 6A–D). This suggests that over-expression of WT or mutant TDP-43 protein impaired normal motor neuron outgrowth, causing axons to depart from their normal routes and to stop short of their target structures.

TDP-43 causes cytoplasmic aggregation, reduced neurite outgrowth and neurotoxicity in vitro

In order to further characterise the effects of TDP-43 on motor axon growth, we transfected the same EGFP-tagged constructs as above into chick primary spinal motor neurons, After 24–96 h in culture, the cells were immunostained with antibodies for the Islet-1/2 motor neuron-specific marker and anti-GFP (in order to visualise the distribution of TDP-43). Analysis of control, EGFP-transfected, Islet-1/2-positive motor neurons after 24 h in culture showed that the EGFP protein was distributed throughout the cell body and neurites (Figs. 7Ai-iii). At 24 h, EGFP-tagged TDP-WT and mutant forms however were restricted to the nucleus of Islet-1/2-positive motor neurons and were not observed to mis-localise to neurites or form aggregates, nor did cellular toxicity occur (Figs. 7 Bi–Biii, Ci–Ciii, Di–Diii). EGFP-transfected cells at 72 and 96 h were viable and healthy and contained EGFP protein throughout the neurites (Figs. 7Aiv,Av). However, at the same time points, we observed increased cell death of neurons transfected with TDP43-WT and mutant forms, reflected by cellular fragmentation and mis-localisation of all TDP-43 isoforms to axons (Figs. 7Biv, Bv, Civ, Cv, Div, Dv). Approximately 25% of cells showed evidence of disintegration and cell death. These results suggest the presence of TDP-43 cytoplasmic aggregates and a toxic effect of wild-type and mutant TDP-43 which may be progressive over time, with a time-course following transfection which is similar to that seen *in vivo*.

To analyse the effects of TDP-43 on cytoskeletal integrity, we performed immunostaining of the transfected cells with anti-NAA to identify and measure neurites. Observation of immunostained cultures at 72 and 96 h demonstrated that surviving motor neurons which expressed wild-type and mutant TDP-43 had shorter neurites than neurons expressing the EGFP control plasmid (Fig. 8A-D). Quantitation suggested that the total neurite length per motor neuron expressing TDP-43 wild-type and mutant constructs was significantly reduced compared to EGFP control-transfected motor neurons (Fig. 8E; $p < 0.05$ for *EGFP* vs *WT*, *Q* and M mutants at 72 h and $p < 0.01$ for *EGFP* vs *WT*, *Q* and M mutants at 96 h). These data suggest that the expression of TDP-43 wild-type and mutant isoforms impairs motor axon outgrowth *in vitro*, mirroring the reduction in axonal length seen *in vivo*.

Conclusions/discussion

In this study, we have analysed the role of TDP-43 in an embryonic chick model system *in vivo* and *in vitro*. We have tested the relative effects of over-expression of wild-type and mutant TDP-43 on motor neuron viability, axon outgrowth and cytoskeletal integrity. Our results demonstrate that TDP-43 causes toxicity to spinal motor neurons;

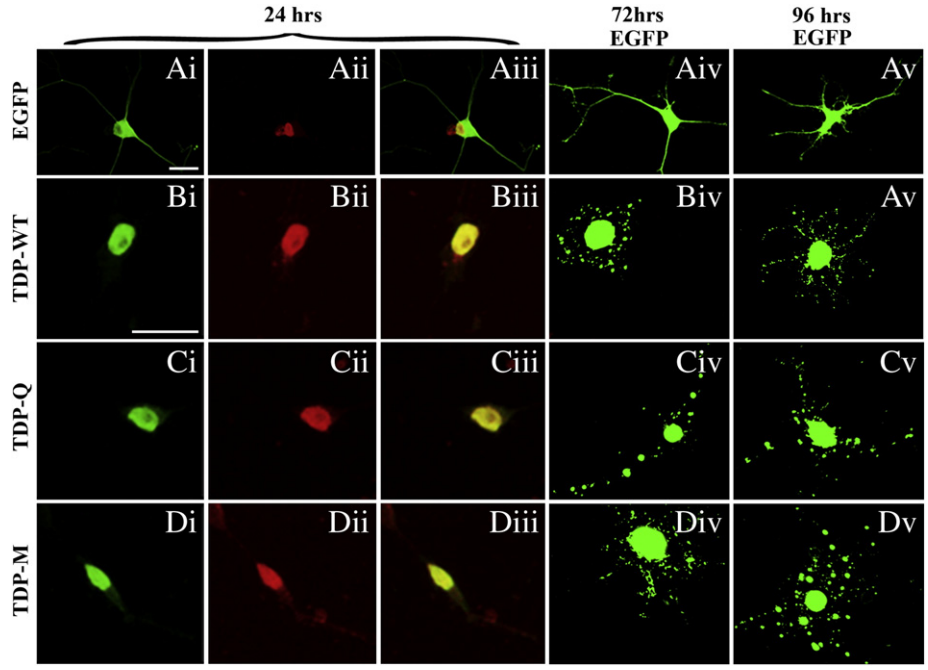

Fig. 7. *In vitro* analysis of the effects of TDP-43. Representative images of chick primary spinal motor neurons transfected with *EGFP* (A), or TDP-43 isoforms *WT* (B), *Q* (C) and *M* (D). It is evident that there is cellular toxicity along with aggregation of TDP-43. Panels (Ai–Aiii, Bi–Biii, Ci–Ciii, Di–Diii) show cells at 24 hour post transfection; EGFP stained in green and red staining represents Islet 1/2. This demonstrates that motor neurons are successfully transfected. Panels (Aiv, Biv, Civ, Div) show cells at 72 hour post transfection. Comparison with *EGFP*-transfected cells (Aiv) clearly demonstrates cytotoxicity and cytoplasmic aggregation of transfected *TDP-43* (*WT* (Biv), *Q* (Civ) and *M* (Div)). Panels (Av, Bv, Cv, Dv) show cells at 96 hour post transfection. Comparison with *EGFP* transfected cells (Av) clearly demonstrates cytotoxicity and cytoplasmic aggregation of transfected *TDP-43* (*WT* (Bv), *Q* (Cv) and *M* (Dv)). Scale bar = 100 μm.

the toxic effects of TDP-43 mutant isoforms were more severe than those of TDP43-WT. *In vivo*, TDP-43 shows cytoplasmic localisation to cell bodies 1–2 days after transfection, and progressive translocation into motor axons after 4 days. Expression of TDP-43 wild-type and mutant proteins in motor axons is accompanied by a truncation of axons en route to their peripheral targets and down-regulation of a neurofilament-associated antigen. *In vitro*, spinal motor neurons which express these isoforms show a similar reduction in neurite length.

TDP43 shows progressive effects in the chick embryo in vivo

Our *in vivo* findings confirm and extend previous work demonstrating neurotoxicity of TDP-43 in the chick spinal cord (Sreedharan et al., 2008). Based on the proportion of dying cells and those showing cytoplasmic mis-localisation of TDP-43 protein, the effects of mutant isoforms were more pronounced than that of the wild-type TDP-43. Our data in conjunction with other published results (Sreedharan et al., 2008) suggest that the neurotoxic effects of TDP-43 are correlated

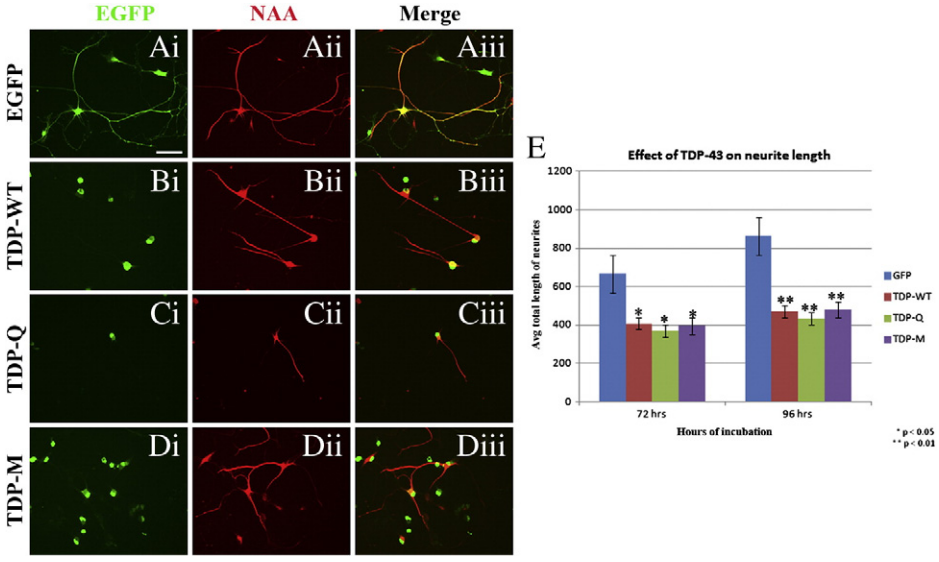

Fig. 8. TDP-43 causes a decrease in process length of neurons *in vitro*. Representative images of chick primary spinal motor neurons transfected with *EGFP* (A), or TDP-43 isoforms *WT* (B), *Q* (C) and *M* (D), cells were analysed after 72 and 96 h in culture for total neurite length. Images represent cells after 96 h in culture. Panels Ai–Di show immunostaining of *EGFP*-tagged constructs (green); Panels Aii–Dii show immunostaining of NAA (red); Panels Aiii–Diii show merge of EGFP (green) and NAA (red). The images are taken at the same magnification in order to demonstrate the decrease in length of neurites in cells transfected with wild-type and mutant TDP-43. (E) Graph demonstrating mean neurite length. Scale bar = 100 μm.

with cytoplasmic aggregation/mis-localisation, which in turn may disturb aspects of the RNA processing function of TDP-43. Expression of mutant TDP-43 in rat cortical neurons led to toxicity after several days in culture, and there was a strong correlation between the cytoplasmic mis-localisation and neuronal death (Barmada et al., 2010). However, it remains an open question whether cytoplasmic localisation per se leads to cell death in our system. For example, in mouse models of TDP-43-mediated proteinopathy, although ubiquitinated cytoplasmic aggregates were observed, these did not contain TDP43, suggesting that disrupted function of TDP43 DNA/RNA binding rather than cytoplasmic aggregation per se is responsible for the toxic effects (Wegorzewska et al., 2009).

In vivo, we also observed a progressive recruitment of TDP-43 protein to axons over time, accompanied by aberrant truncation and defasciculation of axon projections. Our *in vivo* findings of motor axon defasciculation are consistent with previous studies in the zebrafish, in which over-expression of mutant TDP-43, and to a lesser extent WT-TDP-43, caused ectopic motor axonal branching phenotypes (Kabashi et al., 2008; Kabashi et al., 2010).

Our *in vivo* data paralleled our *in vitro* findings demonstrating that there is a decrease in the axonal length 72 and 96 hour post-transfection of WT and mutant TDP-43 forms. These data correlate with recent studies, in which mutant TDP-43 was found to inhibit neurite outgrowth (Duan et al., 2011), and over-expression of wild-type (WT) and mutant TDP43 causes toxicity in motor neurons (Fallini et al., 2012; Kabashi et al., 2010).

The cytoskeleton is a target of TDP-43 dysfunction

In addition to the effects on axon growth we document here, we found that TDP-43 over-expression is associated with a down-regulation of NAA. Whereas we did not quantitatively assess NAA levels in vitro in the presence of TDP43 overexpression, a reduced level of neurofilament expression is a likely correlate of the reduction in axon length which we observed. Our results contribute to a growing body of evidence suggesting that TDP-43 functions to regulate the cytoskeleton, and that loss of this regulation is an important feature of TDP-43 pathology. One mechanism to explain this dysregulation of the cytoskeleton is via aberrant processing or trafficking of RNAs which are normally bound to TDP-43. Studies in which TDP-43 was shown to be trafficked into motor axons, and to colocalise with several mRNA-binding proteins are also consistent with this view (Fallini et al., 2012). Indeed, TDP-43 has been shown to bind directly to the 3′ UTR of the low molecular weight (68 kDa) neurofilament mRNA (NFL) stabilising it to prevent degradation (Strong et al., 2007). Conversely, in ALS the expression of the NFL mRNA is suppressed or preferentially sequestered to stress granules (Strong et al., 2007; Volkening et al., 2009). Over-expression of mutant TDP-43 in *Drosophila* causes severe motor deficits and structural defects at the neuromuscular junctions (Feiguin et al., 2009). Alterations in the organization of synaptic microtubules correlate with reduced protein levels in the microtubule associated protein futsch/MAP1B (Godena et al., 2011). In TDP-43 transgenic mice over-expressing TDP-43 A315T or G348C mutations, it has been shown that there are perikaryal aggregation and axonal aggregation of intermediate neurofilaments (Swarup et al., 2011). Additional cytoskeletal targets of TDP-43 in the chick embryo remain to be characterised.

To conclude, our data suggest that TDP-43 regulates axon outgrowth and the integrity of the cytoskeleton, and that dysfunction of TDP-43 contributes to the pathology of ALS. We here demonstrate the advantages of the chick embryo system, in allowing characterisation over several embryonic stages of the progressive recruitment of TDP-43 wild-type and mutant protein to motor axons These observations suggest that early stages of chick development might encapsulate an acute model of changes taking place over a longer timescale in mice or in humans.

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